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Contents

ORIGINAL ARTICLES

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Under the above caption will be published from time to time comments, criticisms and suggestions on technical procedures; minor contributions such as laboratory aids and short cuts which are not considered sufficiently important to warrant a formal paper; and queries.

Obviously comments and criticisms should be signed; queries should be signed but names will be withheld on request; full credit will be given those who contribute laboratory aids, short cuts and the like.

An attempt will be made to obtain answers from authoritative sources to the queries submitted. It must be emphasized that the views expressed in this department are not the opinions of any official body.

THE OXIDATIVE MICRO-ESTIMATION OF BLOOD LIPIDS*

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There appear to be three reasons why the differential lipid analysis of blood has not been generally adopted. First, satisfactory methods for estimating lipids other than cholesterol have appeared only within the last five or ten years; second, the technique of lipid micromethods cannot be mastered within a short time; and third, the practical value of the additional information provided by the complete analysis is not generally appreciated. Whether or not the advantages to be gained from the complete analysis justify the additional labour involved must, of course, be the decision of each person confronted with the question. Categorically these advantages are: first, technical analytical errors rarely appear in all of a group of analyses performed on the same sample of blood so that, if need be, a diagnosis may be usually reached by the aid of one differential lipid analysis, second, variations in the amount of lipids other than cholesterol form a part of the clinical-pathological picture equally if not more important than the changes in cholesterol and the added information provides the satisfaction of knowing more of what is taking place, and third, which is most important, the extra information may provide a clue to the diagnosis which would not appear in the estimation of cholesterol alone.

There is prevalent a misconception that the amount of all lipids of blood always vary simultaneously in the same direction when variation occurs so that the determination of one lipid, say cholesterol, provides an index for the entire group. This is not always the case and, indeed, is true for the most part only within

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the range of normal values.[10]   The earliest evidence of an impending lipemia, and at any time the most marked change, is an increase in the amount of plasma neutral fat[12].   There are, however, other types of lipemia, e.g. that following abortion in women[11] and in hypothyroidism[17], in which increases in the amount of the cholesterol fractions and of phospholipid are the distinctive features.   A marked increase in plasma free cholesterol is the characteristic change in the lipemia of obstructive jaundice[19].   Normal values for plasma neutral fat with low normal values for other lipids suggest an impending lipopenia which, when fully developed, is the antithesis of a lipemia and in man is the characteristic blood finding in fever[3], hyperthyroidism[16], parenchymatous hepatic disease[18], and congestive heart failure[19]. These characteristic changes are not revealed if total cholesterol alone is determined.

Having decided to include a differential lipid analysis in the laboratory schedule, the next question is what methods shall be adopted.   Originally confronted with this problem, the author[2] selected the oxidative microtechnique because by it all of the established groups of lipids in blood could be determined by the same analytical principal—isolation followed by oxidation with chromic acid.   This has several advantages.   Anyone familiar with the literature on lipids must have concluded that the technique is involved and difficult to master since practically every author describing his initial experience with lipid chemistry finds it necessary to add some modification of published methods. With the oxidative procedure, the initiate conserves time by having to master but one general technique and then a few additional maneuvres necessary to adapt this technique to the estimation of each individual lipid.   Since much of the necessary apparatus is used in the determination of all of the various lipids, a minimum of special equipment is required for each procedure. In any scheme of differential lipid analysis, many values have to be calculated from two or more other values; using the oxidative method, the initial figures derived from the analysis are all in terms of the same units, cc. of 0.1 N potassium dichromate, which facilitates calculation.   The method is saving of time and steps

because all of the related apparatus can be conveniently assembled in a relatively small space and a system of rotation and sequence worked out whereby a large number of analyses may be done in a minimum of time. Six complete analyses may be done in one day and the arrangement most economical of time is 18 complete analyses done on two successive days, the beginning of analysis on one group of six extracts overlapping the ending of that on the previous group of six. Usually several weeks of training are necessary for the successful manipulation of the methods and the technique is definitely not one for those who are impatient of detail.

The selection of a sample of blood. A carefully weighed decision must first be made regarding the treatment of blood prior to extraction of lipids, regarding what volume shall be measured for extraction and which or how many of whole blood, plasma, serum, red blood cells or white blood cells shall be analyzed. Lipids are unequally distributed between cells and plasma of blood and in diseases where changes occur, these changes usually take place in plasma. In diseases involving the blood leucocytes marked changes, occasionally sufficient to influence lipid values determined in whole blood[5], may occur and must be recognized. The lipid content of the red blood cells is relatively stable but there too changes may occur[3]. In routine analyses on hospital or dispensary patients an analysis of plasma is usually sufficient but when interesting cases arise and when research is to be done, samples of both plasma and red blood cells should be analyzed. If there is any indication that the white blood cells are involved, they too should be included in the investigation. The analysis of whole blood only should now be discarded except in especial instances, presenting as it does indefinite composite values affected by changes in plasma, erythrocytes, and leucocytes.

The anticoagulant. Anticoagulant salts, such as the oxalates, citrates, cyanides, and fluorides of sodium, potassium, and ammonium produce by osmosis an immediate shrinkage of the red blood cells with dilution of plasma lipids and concentration of cellular lipids[20,21]. This lasts for 4–8 hours after which the initial distribution of water and lipids begins to return and is re-estab-

lished in 24 hours or so.   Thus when anticoagulant salts are used, samples of blood should be centrifuged and extracts of plasma and, if desired, of cells made as quickly as possible after removal from the patient.   Results so obtained should not be compared with values determined in serum or heparinized plasma but only with results where anticoagulant salts were used in the same concentration and extracts prepared within the same length of time after blood had been removed from the patient.   If these precautions are observed anticoagulant salts may be used, especially for routine work.

The several objectional features of anticoagulant salts may be eliminated by using heparin, hirudin, defibrination, or serum. The use of serum has the disadvantage that analysis cannot be made of the red or white cells but it is suitable for routine work. Defibrination frequently results in damage to the red blood cells with consequent hemolysis and is not recommended for routine work.   Hirudin is fairly satisfactory but usually more of the available preparations of it is required than of heparin.   Heparin has proven an ideal anticoagulant for more detailed work[21]. Heparinized blood may be kept in the ice box for two or three days without any appreciable change taking place in the distribution of lipids between cells and plasma.   Larger amounts of heparin are necessary to prevent coagulation of blood in diseases in which the clotting power is increased as in pneumonia for instance.

The aliquot of blood.   The amount of blood measured for analysis should be varied with the suspected lipid content if only one differential analysis is to be done.   When the lipid content of plasma is suspected of being normal or nearly so, a satisfactory complete analysis can be made on 3 cc. of plasma.   If plasma is suspected of being markedly lipemic, 2 cc. should be taken and if very low values are anticipated, 4 cc. are preferable.   Satisfactory analysis of the red blood cells can almost always be obtained with 2 cc. of erythrocytes.   The white blood cells are weighed and 250 mgm. are usually the minimum capable of giving a complete analysis.

Fasting.   Fasting probably need not be insisted upon in

patients taking a normal balanced diet such as prevails in most hospitals. Normal persons and patients with all diseases so far investigated do not show significant diurnal variations in the lipid content of plasma under these conditions[14] but certain changes may occur in the red blood cells[24]. The older conception that meals had a marked effect on the level of lipids in blood was based upon experiments in animals in which relatively tremendous amounts of fat were ingested. In normal man, a consistent and significant post-prandial lipemia is not produced until the amount of ingested fat reaches 200 gm., an amount nauseating and even emetic to most persons especially on an empty stomach as shown by Man and Gildea (see[4]). However, diurnal variations have been investigated thoroughly in very few conditions and where no definite information is available it is desirable to request blood from fasting patients.

Preparation of extracts. Older methods of extracting lipids from blood required continuous extraction with alcohol or ether or other suitable solvent in a special apparatus for 12 hours or more. Much simpler is the Bloor method in which blood is pipetted slowly and with shaking into a relatively large volume of a mixture of 3 volumes of 95 per cent alcohol and one volume of diethyl ether, both redistilled before use, and then bring the whole to a gentle boil by immersing the flask in hot water. Certain authors have been unable to obtain complete extraction by the Bloor method but it has been recently shown[6] that the difficulty lay in insufficient dilution of blood in the solvent. If aliquots of plasma or of serum are pipetted slowly and with shaking into at least 20 volumes of the alcohol-ether mixture, extraction is complete without heating. This is the method of extraction by cold dilution and is the simplest method yet devised of obtaining complete extraction of lipids from blood. Extraction is done in an Erlenmeyer flask which may then be corked until a suitable time arises to filter and analyze the extract. Whole blood requires a somewhat greater dilution and at least 25 volumes of solvent should be used.

The red blood cells must be hemolysed with an equal volume of distilled water before extraction.[7] A convenient method is to add 2 cc. accurately measured of red cells to 2 cc. approximately of distilled water in a 125 cc. Erlenmeyer flask, shake until hemolysed and then add quickly 50 cc. of the alcohol-ether mixture and shake vigorously for a few minutes. On standing the precipitated proteins will clump together but this may readily be broken up with a cleaned glass rod prior to filtering. The extract of the red cells so prepared must not be heated before filtering since heat causes colored decomposition products to dissolve in the solvent and these contaminate the subsequent analyses and give false high lipid values especially for neutral fat.

The method of separating red cells from blood is as follows. A sample of preferably 15 cc. of blood is centrifuged at about 2500 revolutions per minute for three quarters to one hour or until the red cell layer is translucent. The cells are now squarely packed and plasma eliminated from the interstices between them. An aliquot of these cells may now be removed and extracted without first washing the cells with saline.[7] If there are 5 cc. or more of red cells in a 15 cc. conical bottom centrifuge tube or 10 cc. or more in a 50 cc. conical bottom centrifuge tube, the 2 cc. aliquot of cells may be removed without previously drawing off the supernatant plasma.[15] The red cell pipette is gently passed, with the top sealed by one finger, through the centrifuged plasma and red cells and 2 cc. of cells are removed from the bottom of the tube. If insufficient cells be present, the plasma must be completely drawn off prior to measuring the aliquot of red cells as otherwise some plasma will be drawn in with the red cells when pipetting.

An alternative method of analyzing the red cells is the indirect method in which the lipid content is determined from that of whole blood and of plasma in conjunction with the hematocrit reading. The indirect method gives somewhat higher values than the direct as described above and the results are much more variable.[8] With satisfactory methods available for direct analysis, the indirect method should now be abandoned.

For the lipid analysis of the *white blood cells*, 30–50 cc. of blood are required with normal leucocyte counts but less will suffice in most of the leukemias.[5] The use of heparin as an anticoagulant facilitates the separation of the buffy leucocyte layer[23] which is removed from the top of the red cells after thorough centrifuging, using a pair of cleaned forceps.[9] The leucocytes with adherent plasma and red cells are placed on a weighed watch glass, freed of as much as possible of the plasma and red cells by strips of cleaned filter paper, weighed, ground with cleaned sand and extracted with alcohol-ether. The estimation of the lipid content of the white blood cells is of practical value especially as a prognostic aid in infective or potentially infective conditions.[22]

Having prepared the lipid extract as above, it may be *made up to volume* and then filtered or filtered into a 100 cc. volumetric flask, the precipitate washed twice with solvent and pressed to force out as much retained solvent as possible and the filtrate made up to volume. Similar results are given by these alternative procedures.[7] The second method of making to volume after filtration has certain theoretical advantages and the practical advantage that Erlenmeyer flasks, in which the proteins are precipitated in the alcohol-ether, are cleaned more readily than long narrow-necked volumetric flasks.

Storage of extracts. Extracts prepared as above may be stored for a week or two, either filtered or unfiltered, on an ordinary laboratory shelf before they are analyzed. They should not be kept under any conditions, either in the light or dark or refrigerator, for longer than a month. When stored for over a month, phospholipids and cholesterol esters will have become appreciably hydrolyzed and the analysis does not indicate the true lipid content of the originial sample of blood.[13]

ANALYSIS OF EXTRACTS

The filtered extract is divided into two equal parts (i.e. if of 100 cc. into two 50 cc. portions), one being placed in a 100 cc. tall beaker and the other completely washed into a 125 cc. Erlenmeyer flask. All glassware must, of course, be cleaned in chromic acid, washed, rinsed and dried before use and it is preferable to have a set of glassware used solely for the purpose of lipid analysis since minute amounts of extraneous matter, especially of soap, invalidates the analysis. The half portion in the Erlenmeyer flask is for the analysis of total fatty acids and total cholesterol and the half portion in the tall beaker is for the analysis of phospholipid and free cholesterol.

Total fatty acids and total cholesterol. The half portion in the 125 cc. Erlenmeyer flask is evaporated to a volume of approximately 20 cc. on an electric hot plate using a glass rod with sharp ends to prevent superheating. Three to five drops of a saturated, aqueous solution of sodium hydroxide are added and the concentrate saponified on a steam bath. Sufficient steam is admitted to the bath to bring about evaporation of the concentrate to a volume of about 1 cc. in an interval of about 1 hour. The proper amount of heating must be carefully determined since too rapid evaporation, in less than 30 minutes, or too prolonged, in over 2 hours, result in low recovery of cholesterol. At the end of saponification the contents of the flask will not be dry but will appear as small waxy flakes in about 1 cc. of watery medium. If evaporation occurs too quickly, more alcohol-ether may be added and heating continued until approximately one hour has passed.

After saponification, 1 cc. of 25 per cent sulphuric acid and 2–3 drops of a suitable indicator such as phenol red are added to set free the fatty acids and to color the aqueous solution. The flask is then placed on the top of the steam bath for a few minutes until patches of condensed water vapour appear on the inside. Then 10–15 cc. of petroleum ether are added and the flask shaken to aid solution of the lipids in the petroleum ether. The petroleum ether solution is decanted from the red aqueous layer into a cleaned 125 cc. Erlenmeyer flask. The aqueous medium is extracted three times more with successive portions of petroleum ether, each portion of petroleum ether being brought to a gentle boil on the steam bath and well shaken before decanting. The petroleum ether should have a boiling point of 40–60°C. If an Analysed Reagent petroleum ether be used, a blank should be run initially to ensure absence of non-volatile, oxidizable matter. If less pure petroleum ether be used, it should be shaken with and allowed to stand in contact with concentrated sulphuric acid and then redistilled, the correct portion of the distillate being isolated and a blank initially run on this as above.

The combined petroleum ether extracts are then made up to a volume of 50 cc. After thorough mixing, half of this (25 cc.) is transferred to a 125 cc. glass stoppered Erlenmeyer flask (the "oxidation" flask) for the estimation of the total fatty acid fraction and the petroleum ether is evaporated off on the steam bath. Once the petroleum ether has evaporated, the flask should not

be left on the steam bath because further heating oxidizes some of the lipids. The last traces of petroleum ether vapour are blown out of the oxidation flask by a gentle stream of air from a compressed air line.   No petroleum ether vapour should be left in the oxidation flask since it may condense and be oxidized when chromic acid is subsequently added.

Exactly 5 cc. of Nicloux reagent are added followed by exactly 3 cc. of approximately 1N potassium dichromate.   Nicloux reagent is prepared by dissolving separately in distilled water 10 grams of silver nitrate and 10 grams of potassium dichromate, mixing the two solutions in a 1 litre Erlenmeyer flask, allowing the silver chromate precipitate to settle out, pouring off the supernatant mother liquor, washing the silver chromate twice with 500 cc. of distilled water which is also decanted from the settled precipitate and dissolving the silver chromate in 1 litre of concentrated sulphuric acid.   At this point a blank is introduced consisting of 5 cc. of the Nicloux reagent and 3 cc. of the 1N potassium dichromate in an oxidation flask.   A blank need not be introduced earlier in this or in the other procedures described below if it be previously determined that no solvent capable of doing so will leave in the oxidation flask on evaporation a non-volatile, oxidizable impurity.

The Nicloux reagent and potassium dichromate are then thoroughly mixed in both oxidation flasks, inserting the glass stoppers and allowing the oxidizing reagents to pass over all of the inside of the flasks.   The stoppers are removed and placed at an angle in the neck of the oxidation flasks which are then placed on a cast iron plate, one half inch thick, in an electric oven and heated at 124 ± 2°C. for 20 minutes, using an interval clock to time the heating period.   The flasks are removed from the oven and 50–75 cc. of ice cold distilled water added to prevent further oxidation of reagents.   The glass stoppers are inserted and the flasks set aside until cooled to room temperature when they are ready for titration.   They must not be titrated when hot or warm.   A description of the titration may be left until the other procedures have been described to this same point.

The estimation of *total cholesterol* may now be described further.   The remaining 25 cc. of the petroleum ether extract of the saponified and acidified lipids are transferred quantitatively to a 125 cc. Erlenmeyer flask, 5 cc. of 0.2 per cent digitonin Merck in 50 per cent alcohol added and the whole evaporated on the steam bath to a volume of about 2 cc.   Variations in the quality of digitonin from different manufacturers have been found[15] to account for variable recovery of cholesterol so that any new supply of digitonin should be tested against a known amount of cholesterol.   During the evaporation, total cholesterol is precipitated as cholesterol digitonide and the end of this step is indicated by the disappearance of the alcoholic odour and by the appearance of patches of condensed water vapour on the inside of the flask.   To dissolve any excess of digitonin, about 10 cc. of distilled water are added and this brought to a boil with gentle rotation on an electric hot plate.   When cool, about 20 cc. of redistilled acetone are added with shaking to dissolve excess lipids other than

cholesterol digitonide. At this stage cholesterol should appear as finely granular flakes of cholesterol digitonide in diluted acetone lying at the bottom of the flask.

The cholesterol digitonide is filtered with suction through a sintered glass filter inserted through a rubber stopper in the mouth of a suction flask. The filters used are of the type "4G4, Schott and Gen., Jena." and should be initially and as needed tested to deliver about 125 drops of filtrate per minute. If stoppered after use, the filters rarely need to be cleaned but when this is necessary allowing 10 per cent sodium hydroxide to gradually pass through the filter over night serves the purpose; after this, of course, the filters must be thoroughly washed with distilled water and alcohol. The cholesterol digitonide precipitate after filtration is washed twice with redistilled acetone and ether, the precipitate being thoroughly mixed with each washing fluid. The filter is then transferred to a steam jacket of copper coils beneath and through which the stem of the filter is fitted snugly into the top of a side arm glass connecting head. The side arm of the connecting head leads to a water suction pump and the base of the connecting head fits over the neck of an oxidation flask, a rubber gasket being used to ensure an air tight joint. The apparatus is so arranged that when suction is begun fluid passes from the stem of the sintered glass filter through the connecting head and into the oxidation flask. With the apparatus so arranged, the barrel of the sintered glass filter is almost filled with redistilled methyl alcohol which is brought to a boil by passing steam through the copper coils. Cholesterol digitonide dissolves in the boiling methyl alcohol and the solution is drawn into the oxidation flask by suction and the filter is washed with two smaller portions of methyl alcohol each of which is brought to a boil before suction is begun. The oxidation flask containing the methyl alcohol solution of cholesterol digitonide is placed on a moderately hot electric plate and the solvent evaporated. Cholesterol digitonide separates rather quickly as a white layer on the bottom of the oxidation flask at the end of the evaporation and the flask must immediately be removed from the hot plate to the steam bath on which the last traces of methyl alcohol and its vapour are removed by a gentle stream of air. Cholesterol digitonide is then oxidized with Nicloux reagent and potassium dichromate as was the total fatty acid fraction except that the heating period in the electric oven is lengthened to 30 minutes instead of 20 minutes. After heating, ice cold distilled water is added and the flasks are ready for titration.

Phospholipid and free cholesterol. The first half of the alcohol-ether extract was used to estimate total fatty acids and total cholesterol; the second half is utilized to estimate phospholipid and free cholesterol.

The second half of the alcohol-ether extract is placed in a 100 cc. tall beaker and evaporated nearly to dryness on an electric hot plate using a glass rod to prevent superheating. Evaporation on the hot plate is continued until a frothing, which occurs toward the end of the evaporation, occurs. The beaker is then removed from the hot plate, the glass rod taken out and washed into

the beaker with a small amount of petroleum ether, and the beaker placed on a steam bath where the remaining liquid is evaporated off. The residue left after complete evaporation must not be further heated because phospholipids are readily oxidized thereby. When evaporation is complete, the residue is extracted four times with small portions of petroleum ether, running the petroleum ether down the inside of the beaker to dissolve any adherent lipid and bringing the petroleum ether to a gentle boil each time on the steam bath. The petroleum ether extract of the lipids is collected in a graduated 15 cc. conical bottom centrifuge tube and the total volume of extract should be about 12 cc. If there is suspended matter present, it should be centrifuged out and the clear supernatant solution of lipids transferred quantitatively to another graduated centrifuge tube.

A glass rod with rough unglazed ends is placed in the centrifuge tube to prevent super heating[9] and the tube is placed in a small amount of water in a beaker on a hot plate and the petroleum ether evaporated to slightly less than a volume of 1 cc. The glass rod is removed and washed into the centrifuge tube with a small amount of petroleum ether, not more than enough to bring the volume of petroleum ether in the centrifuge tube over 1 cc. To the concentrated petroleum ether extract are added 7 cc. of acetone previously dried over calcium chloride and redistilled. 0.1 cc. of 30 per cent magnesium chloride in 95 per cent alcohol is added, the centrifuge tube gently rotated to mix its contents and let stand for at least 20 minutes to permit of the precipitation of the phospholipids. The tube is then centrifuged at about 1500 revolutions per minute for 5–10 minutes. The clear supernatant acetone solution of lipids other than phospholipid is quantitatively poured into a 125 cc. Erlenmeyer flask containing 5 cc. of 0.2 per cent digitonin in 50 per cent alcohol and evaporated to nearly dryness, free cholesterol precipitating as cholesterol digitonide and being treated as from this point in the procedure for total cholesterol.

The tube containing the acetone-magnesium chloride precipitate of phospholipids and freed of supernatant acetone solution is gently blown out with a stream of air to remove the last traces of acetone vapour. The phospholipid precipitate is then dissolved in 10 cc. of freshly redistilled, peroxide-free ether saturated with distilled water. This moist ether should be tested each time used, with acid potassium iodide, to ensure the absence of peroxides of ether which oxidize phospholipids. Solution of the phospholipids in moist ether is aided by stirring with a cleaned glass rod. On standing, magnesium chloride dissolves by selective preference in the water of the moist ether and separates as a drop or two of aqueous layer at the bottom of the centrifuge tube. The supernatant ethereal solution of phospholipids is completely drawn over into an oxidation flask through an inverted U-tube, one end of which passes into the centrifuge tube and the other end through a cork stopper in the mouth of the oxidation flask, a second glass tube passing through the same stopper and leading to a suction pump. After drawing off with suction the ethereal solution of the phospholipids the inverted U-tube is washed into the oxidation flask with repeated small portions of redistilled ether. The oxidation flask is then

placed on the steam bath and the solvent evaporated off, the last trace of vapour being removed by a gentle stream of air. The residue of phospholipid is oxidized with Nicloux reagent and potassium dichromate and heated for 20 minutes as in the oxidation of the total fatty acid fraction.

Titration. Four oxidation flasks with corresponding blanks are now ready for titration containing respectively the oxidized total fatty acid fraction, total cholesterol, free cholesterol and phospholipid. In actual practise usually more than one of each of these is determined at the same time, six and one blank of each lipid being a convenient group. To each oxidation flask in turn are added 10 cc. of freshly prepared, iodine-free, 10 per cent potassium iodide solution and the iodine so liberated is quickly titrated with standardized 0.1N sodium thiosulphate using a few drops of a 1 per cent starch emulsion as an indicator. The cubic centimeters of the standardized thiosulphate required in each oxidation flask are subtracted from the corresponding blank and the difference indicates the cubic centimeters of potassium dichromate of the same normality required to completely oxidize the lipid present. This difference should not be more than two thirds of the blank titration, otherwise some lipid may not have been completely oxidized. If the contents of the oxidation flask are green before titration, too large an aliquot has been taken for analysis and the procedure must be repeated using a smaller aliquot.

Calculation of results. The aliquot of blood used, the standardization value of the sodium thiosulphate and the differences in cubic centimeters of this thiosulphate required for each lipid are all recorded. A table is then prepared below these results to receive the calculated lipid values as follows:

Total lipid...
Neutral fat..
Total fatty acids..
    a. Phospholipid fatty acids....................................
    b. Cholesterol ester fatty acids..............................
    c. Neutral fat fatty acids....................................
Total cholesterol..
Ester cholesterol..
Free cholesterol...
Phospholipid...

Using the initials of each word makes a convenient quick method of recording the above table.

The first lipid calculated is total cholesterol. The cubic centimeters of thiosulphate representing the amount of this lipid are multiplied by the normality standardization value of the thiosulphate which brings the figure into terms of exactly 0.1N sodium thiosulphate or potassium dichromate. This latter value is divided by 10.62 giving a figure which represents milligrams of total cholesterol present in the aliquot analyzed since 1 mgm. of cholesterol digitonide requires 10.62 cc. of 0.1N potassium dichromate for its complete oxidation. Two things are done with the figure thus obtained: first it is multiplied by 3.92 giving a value which is noted separately and which will be sub-

tracted from the cubic centimeters of exactly 0.1N potassium dichromate required for the total fatty acid aliquot (1 mgm. of cholesterol—not cholesterol digitonide—requires 3.92 cc. of exactly 0.1N potassium dichromate for its complete oxidation and total cholesterol is present along with total fatty acids in the total fatty acid aliquot). Secondly, the milligrams of total cholesterol found above are divided by the cubic centimeters of blood in which they were contained (that is in one quarter of the original aliquot of blood) and multiplied by 100 giving the milligrams per cent or milligrams per 100 cc. or milligrams per 100 grams if weighed, of total cholesterol present in blood which is noted on the table opposite "Total cholesterol."

Next the cubic centimeters of thiosulphate equivalent to lipid present in the total fatty acid aliquot are multiplied by the standardization value of the thiosulphate and from this value are subtracted the cubic centimeters of exactly 0.1N potassium dichromate required to oxidize the total cholesterol present, i.e. the value calculated above. The difference represents cubic centimeters of exactly 0.1N potassium dichromate required to oxidize the total fatty acids and is divided by 3.61 to obtain milligrams of total fatty acids, 3.61 being the number of cubic centimeters of exactly 0.1N potassium dichromate required to oxidize completely 1 mgm. of the usual 16 and 18 carbon chain fatty acids comprising the bulk of fatty acids present in blood. The milligrams of total fatty acids are then divided by the amount of blood in which they were contained (one-quarter of the original aliquot of blood) and multiplied by 100 giving the milligrams per cent of total fatty acids which figure is noted on the table of values.

Phospholipid is then calculated. The titration difference is multiplied by the standardization value of the thiosulphate and divided by 3.0 which is the number of cubic centimeters of exactly 0.1N potassium dichromate required to completely oxidize 1 mgm. of lecithin or cephalin. The resulting number of milligrams of phospholipid is divided by the amount of blood in which it was contained (one-half of the original aliquot of blood) and multiplied by 100 to give the milligrams per cent of phospholipid which figure is recorded opposite "Phospholipid" on the table of values. Two-thirds of this value are calculated as "Phospholipid Fatty Acids" since on the average two-thirds of the weight of most types of lecithin and cephalin found in blood are fatty acids.

Free cholesterol is calculated by multiplying the titration difference by the standardization value, dividing by 10.62 and by the cubic centimeters of blood containing this amount of free cholesterol (one-half of the original aliquot of blood) and multiplying by 100 to give the milligrams per cent of free cholesterol which latter figure is recorded on the table.

The remaining calculations are made from the figures now present on the table of values. The figure for free cholesterol is subtracted from that of total cholesterol to give the value of "Ester cholesterol." On the average, cholesterol combines with 67 per cent of its weight of fatty acids to produce cholesterol esters so that the value of "Cholesterol ester fatty acids" is now recorded as 67 per cent of the value for ester cholesterol. The values of the phospholipid

fatty acids and the cholesterol ester fatty acids are next added together and the sum subtracted from the value of the total fatty acids giving a figure which is recorded as "Neutral fat fatty acids." From this latter figure, "Neutral fat" is calculated by dividing by 95 and multiplying by 100, since 95 per cent of glycerol tristearate, trioleate, etc., is fatty acid. Neutral fat fatty acids really represent residual fat which is probably mostly neutral fat although some at present undescribed lipids of blood may later be found to comprise part of this fraction. Finally, "Total lipid" is calculated as the sum of neutral fat plus cholesterol ester fatty acids plus total cholesterol plus phospholipid. ___

COMMENT

For further details on the technique as described above, the derivation of the various factors and other relevant data, earlier papers should be consulted in which reference is given to the studies of Bloor, Bang, Yasuda, Okey and others from which the present methods were derived[2,4,6-9,13,20,21,23]. If it is not desired to estimate all lipids as herein listed, the technique should be followed as far as and including the preparation of extracts and then appropriate steps taken from the described procedures to estimate that lipid or those lipids which are to be determined.

Certain analyses may require certain modifications of technique. For example, the estimation of very small amounts of phospholipid in a large volume of extract and with much extraneous matter or other lipids present, is facilitated by certain changes in technique[1]. An approximate, rapid method of estimating human plasma total lipid may be made by determining the lipid present in the total fatty acid aliquot and dividing the cubic centimeters of exactly 0.1 N potassium dichromate so found (without subtracting the total cholesterol present) by 3.25, then by the aliquot of blood containing this amount of lipid and multiplying by 100[14]. The method of estimating iodine numbers of the various fatty acids has not been described but the technique may be found in an earlier paper[2].

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A MICROMETHOD FOR THE DETERMINATION OF TOTAL AND FREE CHOLESTEROL*

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The procedure to be described is that of Schoenheimer and the author[5] with a few minor modifications which have somewhat shortened and simplified the original technique. In principle it consists in the precipitation of cholesterol with digitonin followed by the application of a color reaction to the precipitate. The isolation of cholesterol as digitonide entirely avoids the errors which are introduced into other colorimetric methods by other chromogenic substances present in fatty extracts. Conditions have been discovered under which cholesterol digitonide is completely insoluble and may be readily isolated by centrifugation. The method is rapid, relatively simple, and is especially designed for serial analyses. It has the great advantage of requiring no quantitative transfers from one vessel to another aside from pipetting an aliquot of the original extract into a centrifuge tube.

PROCEDURE

Reagents

1. Acetone (redistilled)—absolute alcohol (1:1).
2. Ether, peroxide free, prepared by washing with a sodium sulfite solution followed by several washings with water. The washed ether is distilled over calcium chloride.
3. Acetone (redistilled)—ether (peroxide free) (1:2).
4. Digitonin solution. One gram of digitonin is dissolved in 1 liter of distilled water and the solution is placed in the icebox for at least 24 hours. The precipitate† which forms is filtered off and the filtrate is concentrated to ap-

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† The nature of this substance, which precipitates only from dilute aqueous solutions, is not known. It forms rapidly in a 0.1 per cent solution but comes down slowly in a 0.2 per cent solution. We have some evidence indicating that it tends to give high values when left in the solution though we have not studied the point extensively. We have employed only Hoffman-La Roche, Inc.,

proximately 500 cc. This is best done by placing the solution in a weighed 1 liter flask equipped with inlet and outlet tubes. The mouth of the inlet tube should be kept about 2 cm. above the surface of the solution. A rapid stream of air, filtered through cotton, is blown, or drawn by suction, through the flask, which is immersed in boiling water. The point at which the required amount of water has been removed is determined with sufficient accuracy by weighing the flask with its contents on an ordinary laboratory balance. The concentration requires 3 to 4 hours. Should a sediment appear in the concentrated solution on standing, it should be removed by filtering.

5. Potassium hydroxide solution made by dissolving 10 grams of pure KOH in 20 cc. of water.

6. Hydrochloric acid, approximately 5 per cent, made by diluting 15 cc. of concentrated HCl to 100 cc.

7. Acetic acid, 100 per cent. We have found the Eastman and Kahlbaum products to be equally good.

8. Acetic anhydride. The product, labeled "acetic anhydride (99–100 per cent)," supplied by the Eastman Kodak Company has been uniformly satisfactory.

9. Concentrated sulfuric acid.

Special apparatus required

1. Volumetric flasks, 5 cc.
2. Funnels, 2.5 cm. in diameter.
3. Filter paper, 4.5 cm. in diameter. The filter paper must be extracted with ether or hot alcohol until completely free of sterols.
4. Stirring rods, approximately 13 cm. long.
5. Preserving jars, either pint or quart sizes, with rubber gaskets.
6. Dropping bottles with ground-in pipettes equipped with rubber bulbs.
7. Conical centrifuge tubes, 15 cc., Pyrex, calibrated at 2 cc., and numbered.
8. Transfer pipettes, for use with rubber bulbs. These pipettes are made by drawing out 8 mm. glass tubing. The total length should be approximately 13 cm., and the tip should be approximately 5 cm. long. The orifice should be large enough to permit rapid filling and emptying.
9. Water bath, equipped to hold 15 cc. centrifuge tubes in the dark at 25°. Any pan of fairly large capacity, fitted with a rack and a thermometer, is suitable. The pan may be placed in a box with a door or simply covered with a dark cloth to exclude light.
10. Zeiss Pulfrich photometer equipped with 5 cm. microcells, or any other color-measuring instrument, of equivalent accuracy, in which cells approximately 5 cm. long and containing not over 2 cc. may be used.

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digitonin which we have found to give uniform results. It is possible, in view of the findings of Schoenheimer and Dam,[4] that other samples of digitonin may not behave similarly.

Procedure for analysis of blood serum

Extraction. Approximately 2 cc. of the acetone-absolute alcohol solution are placed in a 5 cc. volumetric flask; 0.2 cc. of serum is run in slowly without shaking during the addition but with vigorous shaking immediately after withdrawal of the pipette. The solvent is brought to a boil (steam bath) with agitation to prevent bumping, and after being cooled to room temperature the contents are made up to volume with alcohol-acetone, mixed thoroughly, and filtered through a small, dry filter. The filtrate should be perfectly clear.

The proportion of serum may be varied widely to compensate for expected abnormalities in cholesterol concentration. For example in analyzing normal rabbit serum 0.4 or 0.5 cc. is usually extracted while the ratio of hypercholesterolemic sera to volume of extract is reduced (usually 0.2 or 0.1 cc. of serum is extracted in 10 cc. volumetric flasks). Should duplicate determinations be desired, the amounts may be increased in approximate proportion. Good results have been obtained with extracts made up in 10 cc. flasks from 0.5 cc. of serum.

Precipitation of free cholesterol.* Two cubic centimeters of the filtrate are pipetted into a centrifuge tube, 1 cc. of the digitonin solution is added, and the solution is stirred thoroughly with a stirring rod which is left in the tube. The tube is placed in a preserving jar, the cover is placed on tightly, and the jar is left overnight at room temperature. The tube is removed to a test-tube rack and the solution is stirred gently to free particles of precipitate which may adhere to the walls of the tube near the surface of the liquid; the stirring rod is removed carefully without touching the upper part of the tube and laid out on a rack so designed that no adherent precipitate is rubbed off;† and the tube is centrifuged until the precipitate is packed tightly enough to permit decantation of the supernatant liquid without appreciable loss of suspended material. (A few small particles usually float at the surface and cannot be centrifuged down. Their loss does not affect the result.) In the original description of the procedure[5] centrifuging for 15 minutes at about 2500 R.P.M. was recommended. The supernatant solution was removed with a fine capillary pipette to which gentle suction was applied. It was found later that our centrifuge (Size 1, International) was running at considerably less than its rated speed. After repairs had been made it was possible to obtain sufficient packing to allow decantation with only 5 minutes centrifuging. The machine is run at close to

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* The procedure is described for a single determination. In practice the average time per determination may be greatly shortened by carrying through a number of analyses (preferably at least twelve) together.

† We use a rack made of heavy wire designed to hold a number of stirring rods. When several samples are run together (as is usually the case), the position of the rods is numbered so they may be replaced in the proper centrifuge tubes.

its maximum speed (about 2800 R.P.M.); there is practically no breakage if Pyrex tubes are used. The time of centrifuging necessary for adequate packing of the precipitate must be determined by each user of the method with his centrifuge.

After decantation the tube is drained for a few moments and the last drop is removed by touching the lip to a clean towel. The stirring rod is replaced in the tube, and the wall of the tube and the rod are washed down with 1.5 to 2.0 cc. of the acetone-ether solution, best added from a dropping pipette with a rubber bulb attached. The precipitate is stirred up thoroughly, the rod is removed to the rack, the tube is centrifuged for 5 minutes, and the supernatant solution is removed as before. The precipitate is washed twice more in the same manner, except that ether instead of acetone-ether is used. The precipitate does not pack so well under ether and it may be necessary to increase the time of centrifuging somewhat to prevent loss at this stage. After the last wash solution has been removed the stirring rod is returned to the tube and left there through the rest of the procedure. The precipitate becomes dry in a short time at room temperature; if it is desirable to hasten the process the tube may be placed in a water bath at about 40° and the last traces of ether may be removed by a gentle current of air. The sample is now ready for color development. It may be stored for several days at this stage.

Precipitation of total cholesterol. One cubic centimeter of the acetone-alcohol extract is pipetted into a 15 cc. centrifuge tube and 1 drop of the KOH solution is added* and stirred into solution with a stirring rod which is left in the tube. The tube is placed in a preserving jar containing a layer of sand about 3 cm. deep which has been heated in a water bath to about 45°. The sand acts as a heat reservoir. The cover is clamped on tightly and the jar is kept at 37–40° (we utilize an ordinary incubator) for ½ hour.

After hydrolysis the tube is cooled, acetone-alcohol solution is added to the 2 cc. mark, and the solution is titrated with 5 per cent HCl (phenolphthalein indicator). The solution is stirred after the addition of each drop, and care is taken to insure that a definite excess of acid is present. Small amounts of HCl have no effect on the precipitation of cholesterol with digitonin, but alkali interferes. One cubic centimeter of digitonin solution is added, the solution is stirred thoroughly (the suspended salt dissolves immediately), the tube is allowed to stand 1 hour or longer, and the precipitate is centrifuged 5 minutes

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* The size of the drop may be varied within wide limits by changing the height of the burette, or pipette tip above the surface of the solution. The closer the tip, the smaller will the drop be as acetone and alcohol vapor lowers the surface tension of the alkali solution. A few trials with blank samples will determine the proper position of the tip. The drop should be of such a size that approximately 0.1 cc. of 5 per cent HCl solution is required to neutralize it; if too large it may be difficult to dissolve it completely.

and washed just as has been described for free cholesterol except that only one ether washing is necessary.

Development and reading of color. The dried precipitate of cholesterol digitonide is dissolved in 1 cc.* of acetic acid. Care is taken, when adding the acid, to wash down the wall of the centrifuge tube in case any particles of digitonide may have adhered to it. Solution may be hastened by warming the tube in a water bath at about 60° and stirring. A few particles of unknown composition sometimes resist solution especially in free cholesterol determinations. They settle rapidly and do not affect the determination if care is taken not to draw them up in the pipette when the sample is transferred to the photometer or colorimeter cup.

Next, the temperature of the water bath is adjusted to 25°, and the tubes† are placed in the bath and left for a few moments to bring them to temperature equilibrium. One is removed, placed in a rack in a small pan, or beaker, containing water at 25°. Two cubic centimeters of acetic anhydride are added, followed by 0.1 cc. of concentrated H_2SO_4, best added from an automatic microburette.‡ The solution is now stirred vigorously (the original stirring rod is still in the tube), and the tube is replaced in the water bath. Another tube is removed to the small bath and the reagents are added as before. The interval between the addition of reagents is so timed that not less than 27 minutes nor more than 37 minutes elapse between the addition of H_2SO_4 and reading. With practice a little over 1 minute is required for a reading; it is possible, therefore, to carry through twenty-four or more determinations in a series. The temperature of the bath is kept at 25° during the procedure.

The Zeiss Pulfrich photometer has been employed exclusively in this laboratory for measuring the color. In reading with this instrument one of the 5 cm. microcells is filled with a blank solution (1 cc. of acetic acid, 2 cc. of acetic anhydride, and 0.1 cc. of H_2SO_4); the other cell is filled with the unknown colored solution, a transfer pipette being used. The density (or percentage transmission§) is read using filter S61, the cells are reversed, and the reading is taken on the opposite side of the instrument (see manufacturer's directions for

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* In case the precipitate appears to be too large to be read, the analysis may be saved by adding double amounts of acetic acid, acetic anhydride, and H_2SO_4. When this step is indicated, can only be learned by experience.

† The procedure of color development and reading is described for a series of determinations (see footnote page 93).

‡ The measurement of 0.1 cc. of H_2SO_4 is difficult since drainage is slow. We have found it best to measure the acid by counting drops. Four drops from our burette are equivalent to almost exactly 0.1 cc.

§ In recent models of the Stufenphotometer density is read directly from the drums. With older models it is necessary to calculate d from the percentage transmission ($d = 2 - \log T$).

details).   The solution is poured out, and the cell is washed out twice (with approximately 0.5 cc. portions) and then filled with the next unknown solution, a clean transfer pipette being used.   The reading is taken as before.

If 0.2 cc. of serum is extracted in a 5 cc. flask, the concentrations of free and total cholesterol in milligrams per 100 cc. of serum are calculated from the equations

$$\text{Free cholesterol concentration} = \frac{1250 \times d}{1 \times K_{mg.}}$$

$$\text{Total cholesterol concentration} = \frac{2500 \times d}{1 \times K_{mg.}}$$

in which d is the density,* l is the length of cell, and $K_{mg.}$ is the extinction coefficient per milligram of cholesterol.   The calculation is based on a long series of determinations[5] on known amounts of cholesterol which showed that the extinction coefficient (K) is proportional to the amount of cholesterol over a wide range (0.025 to 1.5 mg.).   The value of K per milligram of cholesterol was found in this laboratory to be 1.450.   It should be determined independently, with pure anhydrous cholesterol, by each operator.

Unfortunately few laboratories are equipped with an expensive photometer and many inquiries have been made concerning the applicability of the procedure to an ordinary colorimeter equipped with a suitable color filter such as Wratten 71a and micro cups and plungers.   At least three difficulties present themselves: (1) Acetic anhydride and acetic acid in open colorimeter cups may irritate the eyes and interfere with reading.   (2) The color in many samples, especially of free cholesterol, is very weak and difficult to read accurately in a colorimeter.   Fitz[2] attempted to circumvent this difficulty by making 10 cc. of extract (using 0.5 cc. of serum) instead of 5 cc. and using 3 cc. of the extract for free cholesterol.   There is no apparent reason why the volume could not be further increased, if desired, to 4 or 5 cc., provided that the amount of digitonin solution is increased in the same proportion.   (3) Although under the conditions described (in the dark at 25°) the color does not change appreciably during a 10 minute period, it is sensitive to light and heat and changes more rapidly in a colorimeter cup, especially in one of the Klett type with the light in the base of the instrument.   Fitz found it necessary, therefore, to change the standard after reading each unknown sample.   Shapiro, Lerner, and Posen[6] evaded this troublesome procedure by using a dilute ink solution as a fixed

standard; it must be standardized each day against a cholesterol solution of known concentration.

Although the author cannot speak from extended personal experience (a few preliminary experiments with the colorimeter[5] indicated that fairly good results were possible), the reports of Fitz and of Shapiro show that satisfactory results may be obtained with the colorimeter if suitable precautions are taken. It would seem that a combination of Fitz's increased amount of extract and Shapiro's fixed standard would be advantageous. From the foregoing discussion it will be evident that the application of the method to the colorimeter should be undertaken only after careful and extended study with solutions of known concentration.

It has not been possible to apply the procedure to any of the photoelectric measuring devices on the market. Most of them employ cells which have a large surface area and, if thick enough (3 to 5 cm.) to give satisfactory light absorption, require large amounts of solution. Cells with a small enough capacity (maximum 2 cc.) are too thin for use in measuring the range of relatively weak color intensities. Recently Weech of this laboratory has designed an apparatus which meets the requirements of the method, and with which satisfactory readings have been obtained. A description of the instrument will appear elsewhere.

WHOLE BLOOD

The procedure for the analysis of whole blood is identical with that for serum; but the author advises against the determination of cholesterol in whole blood by this or any other procedure except perhaps in special research problems. The best available evidence indicates that red cells contain only free cholesterol in quite constant amount[1]; changes in pathological conditions have not been proved to occur. By contrast the cholesterol of serum is largely combined with fatty acids and varies within wide limits in health and still more in certain diseases. There is no evident relation between the cholesterol of red cells and that of serum and the analysis of whole blood is comparable to the determination of cholesterol in a mixture of two different tissues, such as brain and adrenal gland.

TISSUES

In principle the procedure is the same for tissues as for serum. A suitable amount of finely divided tissue is introduced into a 5 cc. volumetric flask and extracted with alcohol-acetone. The chief difficulty arises in obtaining uniform samples. The amount needed is small and the chance of taking a non-representative aliquot is correspondingly increased.

The following technique has worked satisfactorily with liver. A piece of glass tubing, 4–5 mm. in inside diameter and about 10 cm. long, is fitted with a plunger of glass rod about 12 cm. long. The rod should fit closely without binding. A small wire rack on which the tube may be placed in a horizontal position on the balance pan is prepared. The portion of liver to be analyzed is cut into small pieces with scissors and ground up to a homogeneous paste in a small mortar. The glass rod plunger is placed in the tube with one end pulled back about 2 cm. from the end and an amount of the liver mash suitable for analysis is worked into the tube with a small spatula. The amount must be learned by experience; for normal livers approximately 5 mm. of the tube should be filled. The tissue adhering to the outside of the tube is wiped off carefully and the apparatus is weighed. The tube is inserted into the neck of a 5 cc. volumetric flask containing about 2 cc. of acetone-alcohol and the tissue is forced out by pushing the plunger down. The plunger is pulled back into the tube and the apparatus is removed from the flask, which is shaken well with a swirling motion at once. A finely divided precipitate should result. If shaking is delayed, the tissue tends to harden into masses which cannot be broken up. The tube and plunger are re-weighed; the difference gives the weight of tissue taken for analysis. From this point on the procedure is the same as that described for serum except that with normal livers only 1 cc. of extract is taken for free cholesterol determination and alcohol-acetone is added to the 2 cc. mark before precipitation with digitonin.

The foregoing procedure should be applicable to other glandular tissues, such as kidney and spleen, which can be ground up to an homogeneous mash. It does not work with brain and muscle.

Preliminary experiments indicate that the homogenizer of Potter and Elvehjem[3] may be useful in the determination of cholesterol in brain. Stable emulsions of this organ are obtained readily and are pipetted for cholesterol determination just as is blood serum. Duplicate determinations on the same emulsion agree satisfactorily.

The method may be applied to the analysis of thin tissue slices,

prepared according to the Warburg technique. The slices are dried on filter paper and weighed directly into dry 5 cc. volumetric flasks. Alcohol-acetone is added and brought to a boil, and the procedure is carried through exactly as described for serum except that 1 cc. of extract, instead of 2 cc., is taken for free cholesterol determination. The method has worked successfully with liver and brain slices.

DISCUSSION

Criteria for the accuracy of the method were presented in the original publication[5]. Later, on the basis of an extensive investigation[7], it was concluded that the procedure gives essentially the same result as the macrogravimetric digitonin method which is generally accepted as the standard of reference. Even more convincing to the author is the fact that in the analysis of several thousand samples of blood serum values for the percentage of free in total cholesterol outside the narrow normal range[8] have been encountered, with very few exceptions, only in the presence of certain pathological conditions.

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AN IMPROVED METHOD FOR THE PHOTELOMETRIC DETERMINATION OF SERUM BILIRUBIN*

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In a previous communication[1], we described a photelometric method for the quantitative estimation of bilirubin in serum. It eliminated the need of color standards which frequently presented difficulties in duplication and even greater difficulties in making comparisons with the unknown. This method seemed more accurate than any other available at that time, even though it was appreciated that some loss of color occurred with the precipitation of the proteins and occasional turbidity in the supernatant fluid.

Since this publication, Malloy and Evelyn[2] have introduced a method which eliminates the above difficulties by carrying out the diazo reaction with diluted serum in a fifty per cent concentration of methyl alcohol, thus leaving the proteins in solution.

We have investigated this new principle and have adapted the method to the Sheard-Sanford photelometer[3]. The technic is as follows:

CALIBRATION OF PHOTELOMETER

Reagents

1. *Stock solution of pure bilirubin.* Weigh 10 mgm. of pure bilirubin† accurately and dissolve in 10 cc. of chloroform.

2. *Solution No. 1.* Dilute 5 cc. of stock solution to 100 cc. with 95 per cent ethyl alcohol.

3. *Solution No. 2.* Dilute 20 cc. of stock solution to 100 cc. with 95 per cent methyl alcohol.

4. *Diazo reagent.* *Solution A.* Dissolve 1.0 gram of sulfanilic acid in 15 cc. of concentrated hydrochloric acid and dilute to 1 liter with distilled water. *Solution B.* Prepare an 0.5 per cent solution of sodium nitrite in distilled water.

The Diazo reagent is then freshly prepared each time by adding 0.3 cc. of Solution B to 10 cc. of Solution A.

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5. *Diazo blank.* Dilute 15 cc. of concentrated hydrochloric acid to one liter with distilled water.

6. Absolute methyl alcohol and 95 per cent ethyl alcohol.

Procedure

Place eight 15 cc. centrifuge tubes in a rack and number them in the following order: 1, 2, 4, 6, 8, 3, 4, 5. With a calibrated pipette, add to tubes 1 to 8 inclusive a corresponding number of cubic centimeters of bilirubin Solution No. 1. To the remaining tubes, add a corresponding number of cubic centimeters of bilirubin Solution No. 2. In the same order, beginning with tube 1, add 8, 7, 5, 3, 1, 6, 5, 4 cc. of 95 per cent ethyl alcohol. Mix gently and then add 1 cc. of diazo reagent to each tube. The solutions are permitted to stand for thirty minutes for complete color development and are then read in the photelometer using distilled water in the standard cell and a green filter with a spectral transmission of 540 mu. The galvanometer readings and the corresponding milligrams of bilirubin are plotted on semilogarithmic paper.

CHECKING OF CURVE

The bilirubin curve was checked by adding known amounts of ethyl alcohol dilution of bilirubin to serum specially treated in order to avoid the occurrence of opalescence.

Procedure

1. To 9 cc. acetone in a 10 cc. glass-stoppered cylinder, add 1 cc. of serum drop by drop and with shaking. Stopper, shake thoroughly, and filter. If the first few drops of filtrate are not clear, pour it through the filter again.

2. Set up three 15 cc. centrifuge tubes. Label one "blank," one "serum," and one "serum plus bilirubin."

3. Into "blank" and "serum" tubes, measure 6 cc. methyl alcohol and into "serum plus bilirubin" tube, measure 5 cc. methyl alcohol.

4. To "blank" tube, add 1 cc. of hydrochloric acid solution and mix and to each of the other tubes, add 1 cc. of diazo reagent and mix.

5. To all three tubes, add 3 cc. of the acetone filtrate obtained in Step 1 above. Mix gently by inversion.

6. To the "serum plus bilirubin" tube, add 1 cc. of bilirubin Solution No. 2. Mix gently by inversion.

7. Allow the tubes to stand thirty minutes for color development. Read in photelometer, using contents of "blank" tube for the Standard cell.

Recoveries made by this method are recorded in table 1.

ANALYTICAL PROCEDURE

A. *Qualitative determination of bilirubin*

1. Overlay 0.3 cc. of serum in tip of centrifuge tube with 0.3 cc. of diazo reagent. The reaction at the point of contact of the two liquids will be nega-

tive, immediate, delayed, or biphasic.   This procedure provides a more accurate reading than that obtained with the original Van den Bergh technic. In addition, the unmixed portion of the serum provides a control color for comparison with the color produced by the diazo reaction.

B. Quantitative determination of bilirubin

1. Set up two 15 cc. centrifuge tubes.   Label one "blank" and the other "serum."

2. To "blank" tube, add 5 cc. of absolute methyl alcohol and 1 cc. of hydrochloric acid solution.

3. To "serum" tube, add 5 cc. of absolute methyl alcohol and 1 cc. of diazo reagent.

4. Dilute 1 cc. of serum to 10 cc. with distilled water.   Mix and add 4 cc. of it to the "blank" and "serum" tubes.

TABLE 1

NUMBER	BILIRUBIN IN SERUM	BILIRUBIN ADDED	TOTAL BILIRUBIN	THEORETICAL	RECOVERY
	mgm.	mgm.	mgm.		per cent
1	1.23	1.0	2.23	2.23	100
	1.23	2.0	3.20	3.23	99.1
2	1.62	2.0	3.68	3.62	101.6
	1.62	3.0	4.60	4.62	99.5
3	0.95	2.0	3.00	2.95	101.7
	0.95	3.0	4.02	3.95	101.8
4	0.82	2.0	2.90	2.82	102.8
5	0.58	2.0	2.51	2.58	97.3

5. Mix gently by inversion and let stand thirty minutes.

6. Read in Photelometer, using Filter 540 mu.   If the galvanometer reading is less than 10, it is advisable for the sake of greater accuracy to dilute both tubes with 10 cc. of 50 per cent methyl alcohol and read again immediately. The bilirubin value must then be multiplied by two.

C. Calculation

The milligrams equivalent of the galvanometer reading is multiplied by 2.5 since 0.4 cc. of serum is used in the determination and this represents milligrams of bilirubin per cubic centimeter of serum.

CONCLUSION

A more delicate, accurate qualitative and quantitative method for the photelometric determination of bilirubin in serum is

described, giving the technic of calibrating the photelometer and of checking the photelometric curve.

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MODIFIED TECHNIC FOR THE DETERMINATION OF HEMOGLOBIN BY THE SHEARD-SANFORD PHOTELOMETER

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In response to many requests by those who are using the Sheard-Sanford Photelometer for the determination of hemoglobins, the following routine method is described, which has proved very accurate in our laboratory.

Collecting tubes are prepared by cutting down 7.5 x 1 cm. tubes to 3 cm. in length. Each evening the approximate number of tubes to be used the next day are made ready by adding one drop of 10 per cent potassium oxalate to each tube and placing it in the incubator to dry over night.

The tube containing the powdered oxalate can easily be carried to the bedside in a shallow paste-board box in which holes of the proper size have been cut.

Blood is collected by ear or finger puncture and each drop of blood is thoroughly mixed with oxalate. The collected blood is brought to the laboratory and 0.05 cc. of it is pipetted into a 6- x $\frac{3}{4}$-inch test tube containing 10 cc. of 0.1 per cent sodium carbonate. After mixing well, by inverting the tube once or twice, the solution is allowed to stand for five minutes to insure complete hemolysis and is ready to read. The adapter or insert is placed in the photelometer cell and the reading obtained.

By this method, the determination of hemoglobin can be made at one's convenience in the laboratory and several examinations can be made at a time.   Between each reading the photelometer cup is simply rinsed well with running water and used again without drying, after carefully draining.   The use of test tubes, of course, is preferable to beakers because of convenience, as well as cost.

PUBLISHED PROCEDURES RECOMMENDED FOR TRIAL

THE ANALYSIS OF CALCIUM IN BLOOD AND OTHER BIOLOGICAL MATERIAL BY TITRATION WITH CERIC SULFATE

C. E. LARSON AND D. M. GREENBERG, J. Biol. Chem. **123**: 199.   1938

Reagents

1. Saturated ammonium oxalate (about 4 grams per 100 ml. of H_2O).

2. 2.0 per cent ammonium hydroxide solution (2 ml. of concentrated NH_4OH, 98 ml. of water).   Saturate with calcium oxalate by agitation with a small piece of solid calcium oxalate, and filter.

3. 0.1 M ceric sulfate.   Dissolve 13.2 to 13.4 grams of anhydrous $Ce(SO_4)_2$ by heating with 20 ml. of concentrated H_2SO_4 and adding water until dissolved. Stir during the process.   Transfer to a 1 liter volumetric flask and dilute to the mark.   Standardize with sodium oxalate or ferrous ammonium sulfate.   Keep ceric sulfate solution from contact with rubber stoppers or other carbonaceous material.

4. 0.005 M ferrous ammonium sulfate (Mohr's salt).   Dissolve 1.96 grams with 9 ml. of concentrated HCl and make up to 1 liter.

5. Phenanthroline indicator.   Dissolve 0.695 gram of $FeSO_4 \cdot 7H_2O$ in a 100 ml. volumetric flask.   Add 1.485 grams of o-phenanthroline monohydrate, stir until dissolved and make up to volume.   Before using indicator titrate 1 ml. with ceric sulfate solution.   Use 2 drops of the neutralized indicator for a titration.

Procedure

1. To 2 ml. of serum add 2 ml. of H_2O and 1 ml. of ammonium oxalate solution.   Let stand for 2 or more hours.

2. Filter through a Kirk-Schmidt[1] microfilter which has a stem 2 inches long.

3. Wash twice with 3 ml. portions of 2 per cent NH_4OH solution pouring washings from the precipitation tube onto the filter.

4. Place the precipitation tube within the suction flask and insert the tip of the filter within it.   Dissolve the calcium oxalate precipitate with three 1 ml. portions of 2 N H_2SO_4.   Wash out the residue with 2 portions of 2 to 3 ml. each of water.

5. To titrate add 2 ml. of 0.01 M ceric sulfate to the tube containing the dissolved calcium oxalate and let stand for 30 minutes.   Add 2 drops of neutralized phenanthroline and titrate back with ferrous ammonium sulfate to the end point.   The color changes are purple → blue green → blue → salmon.   The end point is the change from blue to salmon upon the addition of 0.01 ml. of 0.01 N ferrous ammonium sulfate.

Calculation

Each milliliter of 0.01 M ceric sulfate used is equivalent to 0.4 mg. of calcium.

REFERENCE

(1) KIRK, P. L. AND SCHMIDT, C. L. A.: J. Biol. Chem. 83: 311.   1929.

ANALYSIS OF URINARY CALCULI

Published in pamphlet form by The Association of British Clinical Pathologists, signed C. D.

Submitted by A. S. GIORDANO

The calculus is first washed with water and then dried in the incubator. It is then weighed and a note made of the gross characters, surface appearance, etc.   A tracing of the outline may be made to provide a record of the size and shape.   Whenever possible the stone should be sawn into two equal halves because this gives a good view of the internal structure and nucleus.   A small hand saw or fret saw serves the purpose.   The resulting powder can be used for chemical analysis.   If possible the material from the center of the stone should be removed for separate analysis.   If the calculus is too small to be sawn in two, it may be ground up in a mortar but this method does not allow for a separate analysis of the nucleus.   Least satisfactory of all is to scrape the surface for fragments to analyse, a procedure which may prove misleading because the outer casing of a calculus is often of a different chemical composition than the main bulk of the stone.

Reagents required.   The following reagents are required for chemical analysis of calculi:

Dilute hydrochloric acid.
Concentrated nitric acid.
Concentrated ammonia.
Dilute acetic acid.
Sodium hydroxide 10 per cent.

Fresh ammonium molybdate (made by dissolving a few crystals in water). Potassium hydroxide 10 per cent.

Lead acetate solution.

Scheme of analysis. 1. Place a little of the powder in a clean test tube and add a few drops of dilute HCl. If effervescence occurs, *carbonates* are present.

2. Place a little of the powder in a crucible and heat over a flame till red-hot, cool and shake into a clear tube. Add dilute HCl. If effervescence now occurs, but was not found before heating, then *oxalates* are present.

Confirmatory tests for oxalates may be carried out as follows: Dissolve some of the original powder in warm HCl, cool the tube and add strong ammonia until alkaline and notice whether or not a precipitate occurs. If a precipitate occurs, the calculus contained oxalates or phosphates which may be distinguished by the addition of nitric acid, oxalates being insoluble and phosphates soluble. If oxalates or carbonates are present, the base may be assumed to be *calcium*.

3. Carry out the murexide test as follows: To a pinch of the powder in a small evaporating dish add two drops of nitric acid and evaporate to complete dryness in the water bath. If *uric acid* or urates are present a red color appears which turns reddish-violet (murexide reaction) on adding dilute ammonia (5 drops of concentrated ammonia to about a test tube of water).

To distinguish between uric acid and ammonium urates place some of the fresh powder in a test tube, add sodium hydroxide (10 per cent) and heat. Examine for the evolution of ammonia by the smell and by a piece of moist litmus paper held at the mouth of the test tube. If ammonia is detected the material contained ammonium urate. If no ammonia is evolved, the material giving the murexide test was uric acid.

4. Dissolve some of the powder in dilute hydrochloric acid. Filter if necessary. Add a few drops of concentrated nitric acid and excess of ammonium molybdate solution and boil. *Phosphates* cause a heavy yellow precipitate.

A confirmatory test for phosphates may be carried out as follows: Dissolve some of the powder in warm HCl; cool the tube and add ammonia until alkaline. If a precipitate occurs this may be due to oxalates or phosphates. The precipitate due to phosphates is soluble in acetic acid but that due to oxalates is not. To distinguish between triple phosphates and calcium and magnesium phosphates treat the powder with potassium hydroxide (10 per cent). If ammonia is evolved triple phosphates are present; if no ammonia—calcium and magnesium phosphates.

5. Other rarer forms of calculi are recognized by burning tests.

(a) *Cystine* burns with a pale blue flame causing a sharp pungent smell. To confirm the presence of cystin dissolve the powder in a few drops of concentrated ammonia, filter if necessary, and allow to evaporate spontaneously. Cystin is recognised by the formation of typical hexagonal crystals. Also boil a pinch of the powder with 10 per cent caustic soda and two drops of lead acetate solution. If cystin is present a black precipitate forms.

(b) *Urostealiths* burn with a pale yellow flame and an odour of resin. They are also easily distinguished by the fact that calculus powder is soluble in alcohol and ether.

(c) *Fibrin* burns with a yellow flame and an odour of burnt feathers. The powder is insoluble in alcohol and ether but soluble in hot potassium hydroxide. When acetic acid is added to the caustic potash solution the fibrin is precipitated with evolution of hydrogen sulphide gas.

(d) *Xanthine*. The powder burns away without a flame. Carry out the murexide test. Residue left after heating is yellow. Add a drop of sodium hydroxide and an orange color develops which becomes red on warming.

URINE CHEMISTRY IN THE DIAGNOSIS OF EMBRYONAL TUMORS

S. E. Owen, Q. G. Polanco, and L. H. Prince, Am. J. Cancer **31**: 613. 1937

1. To 40 cc. of fresh morning urine or a preserved twenty-four hour specimen add hydrochloric or acetic acid until acid to litmus. Any precipitate such as albumin is removed by centrifugation.

2. Pour specimen into 200 cc. of 95 per cent alcohol. Chill flask and centrifuge.

3. Wash the wet residue with about 30 cc. of ether or acetone to remove estrin-like substances.

4. Dry. (May be stored in ice chest for long periods.)

5. Dissolve the dry powder in 8 or 10 cc. of distilled water. Any insoluble residue is removed and the remaining solution employed for the test.

6. To each tube containing 1.0 cc. of the above extract add 0.15 cc. of 3.0 per cent hydrogen peroxide. Shake and allow to stand for ten minutes.

7. Add 0.3 cc. of 1.0 per cent aqueous solution of phenylhydrazine hydrochloride. Mix well for five minutes.

8. Add 0.3 cc. of an aqueous solution of methyl cyanide. Mix thoroughly and allow to stand for five minutes.

9. Add 0.3 cc. of concentrated hydrochloric acid. Tubes are shaken and placed in a boiling water bath for twenty to twenty-five minutes.

Readings are made on the warm tubes and reactions classed as follows:

Very strong positive $(+ + + +)$, liquid is russet to brown, containing a thick flocculent brick-dust precipitate which tends to form aggregates.

Strong reaction $(+ + +)$, liquid straw-colored with a flocculent brick-dust precipitate.

Positive $(+ +)$, liquid yellowish and small amount of brown precipitate.

Weak positive $(+)$, yellowish liquid and tan precipitate.

Suspect $(\pm)$, yellowish liquid and sparse tan precipitate.

Negative $(-)$, colorless to light yellow clear liquid and no precipitate.

The tubes may be put in the ice-box overnight and read when cold. The color of the precipitate is more apparent when it has settled.

The test is for urinary prolans.   In 44 cases of teratoma testis giving positive biological test, the chemical test was positive in 39.

In 26 cases where the urinary prolans were 400 or more mouse units the chemical test was positive in 25.

In 11 urines from pregnant women the chemical test was positive in all.

DETERMINATION OF THE ICTERIC INDEX BY THE ACETONE METHOD

ROBERT A. NEWBURGER, J. Lab. & Clin. Med. **22**: 1192.   1937

To 1 cc. of serum in a graduated centrifuge tube is added 1 cc. of colorless, redistilled acetone or in deeply jaundiced serums 3 parts of acetone are added. The solution is mixed, allowed to stand for 5 minutes, centrifuged and the supernatent fluid is pipetted into a standardized test tube and compared with the standards.   The standards of from 1 part to 10 parts in 10,000 potassium bichromate in distilled water are required.   These are placed in standardized test tubes and a drop of 0.1 N HCl added to each.

Calculation: Icteric index = number of standard used × dilution of original serum.

Values for icteric index above five obtained by the acetone method are regarded as abnormal though occasionally a normal serum will show an icteric index one or two units higher.

The authors claim the following advantages: The ease with which it is performed, its accuracy, and that hemolysis is eliminated as a source of error.

ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

CLEANING GLASSWARE.   Dr. Margaret Hudson of Tulsa, Oklahoma writes: "Have many laboratories discovered the value of the washing powder marketed under the name of Dreft?   It is advertized as hymolal salt, not soap.

We used it first for the cleaning of microscope slides, that ever-present bugbear of the laboratory.   We keep a jar of a heavy solution of it in front of each microscope and slides are dropped into it as used.   A strong solution is clear when cold, not a gelatinous mass like soap.   When filled, the jars are drained and the slides dropped, one at a time, into a fresh strong hot suds of the same powder and soaked for a few minutes or boiled if sterilization is desirable. After cooling the slides are washed in this solution, rinsed and dried.   Slides so cleaned may be used for making blood smears with perfect satisfaction.   The oil and stains are dissolved completely without clouding the glass.   It only takes a few minutes to clean a box of slides and they are as clear as when new.

We have adopted Dreft for use in washing all our glassware. Its advantage lies in that it rinses away easily and completely. Glassware dried in the oven sparkles like crystal." Dreft is distributed by the Procter & Gamble Company.

MAYER'S ALBUMIN FIXATIVE. Referring to a note in this department in the January 1938 issue concerning the preparation of the above mentioned reagent Mr. Oscar W. Richards, Research Biologist of the Spencer Lens Company, Buffalo, writes: "For a number of years in various laboratories I have found the following method to be very simple: The whites are separated from the yolks of the eggs and chopped for a few minutes with scissors. The albumin is then poured onto a fluted filter paper and allowed to filter into the container. We changed the filter papers once a day. After the filtration has been completed an equal volume of glycerine is added and whatever preservative one chooses. In warm weather we have found it advantageous to put a few crystals of thymol in the bottom of the bottle which prevents bacterial growth as the albumin filters into it."

BLOOD DIASTASE IN ACUTE PANCREATITIS AND PERFORATING PEPTIC ULCER INVOLVING THE PANCREAS. Probstein, Gray, and Wheeler (Proc. Soc. Exper. Biol. & Med. **37**: 613. 1938) in a study of the blood diastase in nine cases of acute perforating peptic ulcer found a marked increase where there was involvement of the pancreas.

They believe that a high diastase in a case of sudden sharp pain in the upper abdomen indicates pancreatic involvement, a moderate increase during the height of the attack tends to indicate a perforating peptic ulcer and a very high diastase while usually associated with acute pancreatitis may mean an ulcer acutely perforating in or near the pancreas. Normal or low values rule out the last two conditions.

For determining the diastase they used the method of Somogyi (Proc. Soc. Exper. Biol. & Med. **32**: 358. 1936). Essentially this consists of incubating 1 cc. of plasma or serum with 5 cc. of 1.5 per cent starch paste and 2 cc. of 1 per cent NaCl for one-half hour at 40°C., deproteinizing by Somogyi's copper method (J. Biol. Chem. **90**: 725. 1931) and determining the reducing matter by the method of Shaffer-Hartmann-Somogyi (J. Biol. Chem. **100**: 695. 1933). The reduction value of the filtrate minus that of the serum represents diastase. The quantity of the enzyme is expressed as the amount of reducing substances in terms of glucose. The average normal figure is 120 with a range of 80 to 180.

A NEW EMBEDDING MATERIAL. Under the name of "Tissuemat" the Fischer Scientific Co. of Pittsburgh, has introduced a new embedding material which is said to be superior to paraffin in that it will not crack or crumble while being cut. For this reason it is said to be especially suitable for the preparation of serial ribbon sections as well as for the cutting of large sections. The preparation comes in granular form and thus, in addition to melting rapidly, can be prepared easily in whatever amounts are convenient or necessary.

The same company presents unbreakable centrifuge tubes made of "Lusteroid" and an inexpensive automatic saline dispenser for use in connection with serological tests.

The Laboratory Construction Company of Kansas City, Missouri has issued a folder descriptive of improved Kjeldahl Nitrogen Apparatus, a copy of which may be obtained on request.

A new Spencer Microtome catalog containing a concise, well illustrated presentation of rotary, sliding and clinical microtomes and their applications is now ready for distribution.   Copy of this catalog will be mailed upon request to Dept. M, Spencer Lens Company, Buffalo, N. Y.